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STRUCTURE-ACTIVITY RELATIONSHIPS AND IMMUNOCHEMICAL STUDIES ON COBROTOXIN

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Kaohsiung Medical College

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December 1972

U. S. ARMY RESEARCH AND DEVELOPMENT GROUP
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Immunochemical Studies on the Tryptophan-modified Cobrotoxin

The two-dimensional structure of cobrotoxin has recently been established and permits a study of structure-activity relationships. Preceding studies on the chemical modification of cobrotoxin suggested that either the intact Tyr-25, Lys-47, or Glu-21 is essential for full activity of the toxin. The importance of the single Trp-residue at position 29 for the lethality of cobrotoxin as well as several neurotoxins isolated from the venoms of sea snakes have been reported. The immunodiffusion of these Trp-modified toxins showed similar precipitin lines to those of native toxins. However, as for inducing the production of antibodies in animals by immunization with these modified derivatives and the detail immunochemical studies on these products have not yet been undertaken.

In this study, the single Trp-residue in cobrotoxin has been converted into N'-formyl kynurenine by ozonization, oxidized to oxindole derivative with N-bromosuccinimide and also modified by reactions with 2-hydroxy-5-nitrobenzyl (HNB) bromide and 2-nitrophenylsulfenyl chloride. An important feature of the investigation is to determine whether the chemical modification would affect on the antigenic specificity of the toxin.

Each modified derivative gave a single fused precipitin line with cobrotoxin on immunodiffusion against either anti-cobrotoxin or anti-HNB-cobrotoxin sera.

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Each modified derivative gave a single fused precipitin line with cobrotoxin on immunodiffusion against either anti-cobrotoxin or anti-HNB-cobrotoxin sera. In heterologous precipitin reactions, no significant decreases in antigenic activity of the modified derivatives were observed when they reacted with anti-cobrotoxin sera. However, a slight decrease of precipitation occurred for ozonized cobrotoxin. In the production of antibodies in rabbits by immunization with these modified derivatives (except for ozonized cobrotoxin), we have obtained 1.3 times more potent antisera in only one-half the time by using HNB-cobrotoxin instead of native toxin. Therefore, HNB-cobrotoxin is superior to native toxin for the production of antibody in animals. These results suggest that although the tryptophan residue in cobrotoxin is essential for lethal toxicity, it may not be involved in the antigenic specificity of the toxin.

ABSTRACT

Immunochemical Studies on the Tryptophan-modified Cobrotoxin

The two-dimensional structure of cobrotoxin has recently been established and permits a study of structure-activity relationships. Preceding studies on the chemical modification of cobrotoxin suggested that either the intact Tyr-25, Lys-47, or Glu-21 is essential for full activity of the toxin. The importance of the single Trp-residue at position 29 for the lethality of cobrotoxin as well as several neurotoxins isolated from the venoms of sea snakes have been reported. The immunodiffusion of these Trp-modified toxins showed similar precipitin lines to those of native toxins. However, as for inducing the production of antibodies in animals by immunization with these modified derivatives and the detail immunochemical studies on these products have not yet been undertaken.

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Each modified derivative gave a single fused precipitin line with cobrotoxin on immunodiffusion against either anti-cobrotoxin or anti-HNB-cobrotoxin sera. In heterologous precipitin reactions, no significant decreases in antigenic activity of the modified derivatives were observed when they reacted with anti-cobrotoxin sera. In the production of antibodies in rabbits by immunization with these modified derivatives (except for ozonized toxin), we have obtained 1.3 times more potent antisera in only one-half the time by using HNB-cobrotoxin instead of native toxin. Therefore, HNB-cobrotoxin is superior to native toxin for the production of antibody in animals. These results suggest that although the Trp-residue in cobrotoxin is essential for lethal toxicity, it may not be involved in the antigenic specificity of the toxin.

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Immunochemical Studies on the Tryptophan-modified Cobrotoxin

I. Introduction

Preceding studies on the chemical modification of cobrotoxin suggested that either the intact Trp-29 (1), Tyr-25 (2), Lys-47 (3), or Glu-21 (3,4) is essential for full activity of the toxin.

Most snake venom neurotoxins whose amino acid sequences have been established contain only one tryptophan residue located at the homologous positions (5). By means of chemical modification, the importance of tryptophan residue for the lethal toxicity of neurotoxins isolated from the venoms of sea snakes have also been reported (6-9). The immunodiffusion of these tryptophan-modified toxins showed similar precipitin lines to those of native toxins. However, as for the inducing the production of antibodies in animals by immunization with these modified derivatives and the detail immunochemical studies on these products have not yet been undertaken.

In this study, the single tryptophan residue in cobrotoxin has been modified by four different reagents and the effects on the antigenic specificity of the toxin were investigated. The modified derivatives although lost almost completely the lethal toxicity, but the antigenic specificity remained unchanged significantly. Among the modified toxins, HNB-cobrotoxin is the most superior to native toxin for inducing the production of antibody in animals.

Abbreviations: HNB-Br, 2-hydroxy-5-nitrobenzyl bromide; NPS-Cl, 2-nitrophenylsulfenyl chloride; NBS, N-bromosuccinimide; HNB-cobrotoxin, NPS-cobrotoxin, and NBS-cobrotoxin are cobrotoxin derivatives which were modified with HNB-Br, NPS-Cl, and NBS, respectively.

II. Materials and Methods

Cobrotoxin was prepared from Taiwan cobra (*Naja naja atra*) venom as previously described (10). Reagent grade of HNB-Br was purchased from Seikagaku Kogyo Co., Japan. NPS-Cl was obtained from Sigma Chemical Co., and NBS from Pierce Chemical Co. NBS was recrystallized from water before use.

Alkylation of cobrotoxin with HNB-Br and ozonization of cobrotoxin in formic acid were carried out as previously described (1).

1. Sulfenylation of cobrotoxin

Sulfenylation was carried out essentially according to the method of Scoffone et al. (11) To a solution of cobrotoxin (50 mg) in 2 ml of 30 % acetic acid a 10-fold molar excess of NPS-Cl in 1 ml of glacial acetic acid was added. The reaction was allowed to proceed at room temperature (27°) for 1 h and the mixture was desalted by passage through a Sephadex G-25 column (2 x 50 cm) equilibrated with 0.2 M acetic acid. The protein fractions were pooled and lyophylized.

2. Oxidation of cobrotoxin with NBS

Oxidation of cobrotoxin with NBS was performed essentially according to the procedure described by Freisheim and Huennekens (12). To a solution of cobrotoxin (50 mg) in 2 ml of 0.1 M acetate buffer (pH 4.0) a 4-fold molar excess of NBS in 1 ml of the same buffer was added dropwise. After stirring at 27° for 30 min the modified toxin was separated from the excess reagent by passage through a Sephadex G-25 column (2 x 50 cm) equilibrated with 0.2 M acetic acid. The protein fractions were pooled and lyophilized.

3. Preparation of antisera

Anti-cobrotoxin serum was prepared in rabbits as previously described (13). Each anti-Trp-modified cobrotoxin serum was prepared by injecting increasing amounts of the homologous modified toxin (except for ozonized cobrotoxin) with Freund's adjuvant (complete) into rabbits weighing 2.0 to 2.5 kg. Sixty μg to 2.5 mg per kg body weight were injected subcutaneously into the right and left thigh alternating at weekly intervals during a period of two months and the animals were bled 9 days after the final injection.

Amino acid analysis, measurements of lethal toxicity and immunological procedures were performed essentially the same as previously described (2,13).

III. Results

1. Chemical modification of tryptophan residue in cobrotoxin

The extent of ozonization was followed by the increasing absorbancy at 320 nm corresponding to the formation of N-formylkynurenine (14). As shown in Fig. 1, with increasing amounts of N-formylkynurenine formed, the lethal toxicity decrease

progressively and finally dropped to 3.1 % of cobrotoxin after ozonization for 8 min. No further changes in the absorbancy at 320 nm and the lethality were observed after 8 min.

The oxidation of tryptophan by NBS leads to decrease in absorbancy at 280 nm. As shown in Fig. 2, the $A_{280\ nm}$ (1-cm light path) value of the mixture decreased as the amounts of NBS increased within 3 molar excess over cobrotoxin and then went up again with a gentle slope. The lethality of cobrotoxin decreased markedly and finally lost almost completely when all tryptophan residue was modified.

For the reaction of cobrotoxin with HNB-Br or NPS-Cl, the extents of modification were determined spectrophotometrically based on the molar absorbance coefficients (ϵM) of 18,900 and 4,000 at 410 nm (15) and 365 nm (11), respectively. As shown in Figs. 3 and 4, the lethality decreased progressively and finally dropped to 6.2 % and 3.1 % of cobrotoxin, respectively, as all tryptophan residue was modified.

2. Characterization of the modified derivatives

As shown in Fig. 5, the absorption spectra of HNB-cobrotoxin and NPS-cobrotoxin, which showed the characteristic peaks at 410 nm and 365 nm, respectively, were practically the same as those of HNB-proteins and NPS-proteins reported by Barman and Koshland (15) and Scoffone et al. (11), respectively. Spectral determination of the extents of modification based on the molar absorbance coefficients of 18,900 and 4,000 at 410 nm and 365 nm indicated that 0.92 mole and 0.98 mole of tryptophan had reacted with HNB-Br and NPS-Cl, respectively.

The amino acid analyses of the modified derivatives showed that, besides tryptophan, all other amino acids remained essentially intact (Table I). However, the derivative oxidized with NBS, in addition to tryptophan, one mole of tyrosine and histidine had also been modified.

3. Antigenic activity of the modified derivatives

As shown in Fig. 6, no significant decreases in antigenic activity of the modified derivatives were observed in the heterologous precipitin reactions when they reacted with anti-cobrotoxin sera. However, a slight decrease of precipitation occurred for ozonized cobrotoxin.

Inducing the production of antibodies in rabbits by immunization with those modified derivatives (except for ozonized cobrotoxin) was carried out. Due to the low toxicity of the modified derivatives, immunizations were started from 60 μg per

kg body weight instead of 6 μg and completed in only one-half time of that with cobrotoxin (Table II). The antibody contents in the pooled immune sera were determined by quantitative precipitin reactions using cobrotoxin as an antigen (Fig. 7). As shown in Table II, the amount of antibody precipitated from anti-HNB-cobrotoxin sera by cobrotoxin was 8.5 per cent more than that of anti-cobrotoxin sera. However, the antibody content in the anti-NBS-cobrotoxin sera was only one-half that of anti-cobrotoxin sera.

4. Neutralizing capacity of antisera

Comparison of specific neutralizing capacity of anti-Trp-modified cobrotoxin and anti-cobrotoxin sera are shown in Table III. It is seen that the relative capacity of the anti-HNB-cobrotoxin sera was 1.3 times higher than that of anti-cobrotoxin sera, but anti-NBS-cobrotoxin sera was only one-third that of anti-cobrotoxin sera. The antibody content and specific neutralizing capacity of anti-NPS-cobrotoxin sera were almost the same as those of anti-cobrotoxin sera.

5. Immunodiffusion

Practically the same patterns of immunodiffusion were observed for both anti-cobrotoxin (Fig. 8-A) and anti-HNB-cobrotoxin sera (Fig. 8-B) against cobrotoxin and Trp-modified derivatives. Each modified toxin gave a single fused precipitin line with cobrotoxin against either anti-cobrotoxin or anti-HNB-cobrotoxin sera, indicating that the all modified toxins were immunochemically homogeneous as cobrotoxin.

The above results indicate that although the single Trp-29 which is common to all neurotoxins isolated from snake venoms is essential for lethal toxicity, it is not essential for the antigenic specificity of the toxin. Therefore, the less toxic HNB-cobrotoxin is superior to native toxin for inducing the production of antibody in animals. In fact, we have obtained 1.3 times more potent antisera in only one-half the time by using HNB-cobrotoxin instead of native toxin.

IV. Discussion

Tryptophan has been assigned an important role in determining and stabilizing the tertiary structure of protein by its interaction with other hydrophobic residues. In this study, four different reagents which have been reported as specific for the tryptophan residues in proteins were used for the modification of the single tryptophan residue in cobrotoxin and the effect on the antigenic specificity of the toxin was investigated. The data from amino acid analyses of the modified derivatives indicated

that these reactions do not affect the other amino acids, except for NBS-cobrotoxin which showed besides tryptophan, one mole of tyrosine and histidine were also modified (Table I). These modifications lead to the almost complete loss of lethal toxicity, indicating that the tryptophan residue in cobrotoxin is essential for the lethality. The results are in good agreement with those of neurotoxins isolated from the venoms of sea snakes (6-9). It is noteworthy that most snake venom neurotoxins contain only one tryptophan residue located at the homologous positions in the sequences (5) and this invariant tryptophan residue plays important role in the structural features for the lethal toxicity of toxin.

It is of interest to note that while the tryptophan residue is essential for the toxic action, it is not involved in the antigenic activity of toxins. In the immunodiffusion study and heterogous precipitin reactions, no significant differences were observed between cobrotoxin and its modified derivatives. Morever, by using HNB-cobrotoxin as an antigen for immunization, we have obtained more potent antisera in only one-half the time instead of native toxin (Table II and III). This strongly indicates that there is essentially no change in antigenic specificity after tryptophan modification.

In comparison of antigenic activity among the modified derivatives, it showed that HNB-cobrotoxin is the most antigenic and NPS-cobrotoxin, NBS-cobrotoxin, and ozonized cobrotoxin follow in decreasing order (Fig. 6 and Table II). These results indicate that the effects of the four specific reagents on the immunological properties of cobrotoxin are somewhate different. The slight decrease in antigenic activity of cobrotoxin after oxidation with NBS or ozonization may be attributed to the change of gross conformation.

Karlsson and Eaker (16) have recently reported that modification of the single tryptophan residue in _siamensis_ 3 toxin isolated from the venom of _Naja naja siamensis_ with HNB-Br resulted in that the toxin polymerizes thereby easily. Trimers and higher aggregates are non-toxic while dimers are slightly toxic. Three monomeric, toxic derivatives were isolated by gradient chromatography on Bio-Rex 70. Thus, they concluded that tryptophan residue is not functionally essential and is a structurally essential group. By immunochemical study on erabutoxins, neurotoxic proteins obtained from a sea snake _Laticauda semifasciata_, Sato _et al._ (17) have recently reported that the formaldehyde-treated erabutoxins are antigenic although they have no lethal toxicity. It is of interest that the modified erabutoxin b is ultracentrifugally monodisperse and has a molecular weight of 68,800, about ten times that of the native toxin. In order to determine whether the HNB-cobrotoxin is also forming a polymer, the molecular weight was estimated by gel

filtration on Sephadex G-50 equilibrated with M/15 phosphate buffer, pH 7.4. In comparison with cobrotoxin and α-chymotrypsin (Fig. 9), all preparations were revealed as a single peak and the α-chymotrypsin (mol. wt., 22,000) emerged in the void volume and HNB-cobrotoxin as well as cobrotoxin in almost the same volume of eluate, indicating that HNB-cobrotoxin is a monomer.

The results from previous studies on the chemical modification of cobrotoxin have suggested that a variety of functional groups are essential for biological activity of the toxin and the changes in lethal toxicity and antigenic activity had occurred concurrently (2,3,18). However, from the result of present study and that of exhaustive fluorescein thiocarbamylation of cobrotoxin through its free amino groups suggest that these modifications resulted in pronounced decrease in lethal toxicity without affecting the antigenic specificity of the toxin (19,20). These results suggest that the antigenic sites of cobrotoxin are different from the active site(s) of toxicity. The similar observations were also made on the sea snake neurotoxins (6,7,17). In fact, these less toxic preparations can be used as a good tool in the production of antibodies in animals.

V. Conclusion

The single tryptophan residue in cobrotoxin has been converted into N-formylkynurenine by ozonization in formic acid, oxidized to oxindole derivative with N-bromosuccinimide, and also modified by reactions with 2-hydroxy-5-nitrobenzyl bromide and 2-nitrophenylsulfenyl chloride. The modified toxins lost almost completely the lethal toxicity and showed that besides tryptophan, all other amino acid residues remained intact. However, the derivative oxidized with N-bromosuccinimide, in addition to tryptophan, one mole of tyrosine and histidine were also modified.

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Tablo I

Amino acid composition of cobrotoxin and tryptophan-modified derivatives

Amino acid	Residues per mole of protein				
	Cobrotoxin	Ozonized cobrotoxin	HNB-cobrotoxin	NPS-cobrotoxin	NBS-cobrotoxin
Aspartic acid	8	8.4	8.2	7.8	7.6
Threonine	8	7.5	7.9	7.6	7.3
Serine	4	3.3	3.9	3.8	3.7
Glutamic acid	7	7.1	7.3	6.8	7.3
Proline	2	2.0	2.3	2.2	1.9
Glycine	7	7.2	7.3	7.1	7.3
Alanine	-	-	-	-	-
Half-cystine	8	8.3	7.9	8.3	8.1
Valine	1	1.0	1.1	1.0	1.0
Methionine	-	-	-	-	-
Isoleucine	2	2.0	2.1	1.8	2.2
Leucine	1	1.0*	1.0	1.0	1.0
Tyrosine	2	1.7	2.1	1.8	0.90
Phenylalanine	-	-	-	-	-
Lysine	3	3.0	3.1	2.9	3.0
Histidine	2	1.7	1.8	1.9	0.93
Arginine	6	6.1	6.3	5.9	6.3
Tryptophan	1	0.0	0.0	0.0	0.0
Kynurenine	-	0.75	-	-	-
HNB-tryptophan	-	-	0.92**	-	-
NPS-tryptophan	-	-	-	0.98**	-

* All values are expressed as molar ratios based on leucine = 1.0.

** Determined spectrophotometrically at 410 nm and 365 nm for HNB-tryptophan and NPS-tryptophan, respectively.

APPENDIX A-2

Table II

Comparison of the production of antibody to cobrotoxin by immunization with cobrotoxin and tryptophan-modified derivatives in rabbits

Antigen	Lethality (%)	Total dose (mg/kg body weight)	Immunization period (days)	Relative antibody content in immune sera (%)
Cobrotoxin	100	5.118	113	100 (2.59)*
HNB-cobrotoxin	6.2	5.970	58	108.5 (2.81)
NPS-cobrotoxin	3.1	5.970	58	90.8 (2.36)
NBS-cobrotoxin	1.6	5.970	58	52.3 (1.35)

* Numbers in parentheses denote the antibody contents in per cent in the pooled immune sera from four rabbits.

APPENDIX A-3

Table III

Comparison of specific neutralizing capacity of anti-cobrotoxin and anti-tryptophan-modified cobrotoxin sera against cobra venom and cobrotoxin

Antisera*	Specific neutralizing capacity (LD_{50}/mg N)**	Relative capacity
Anti-cobrotoxin	16.1	1.0
Anti-HNB-cobrotoxin	21.7	1.3
Anti-NPS-cobrotoxin	15.5	0.95
Anti-NBS-cobrotoxin	5.6	0.35

* The pooled antisera from four rabbits were used in each measurement.

** The amounts of 1 LD_{50} for cobra venom and cobrotoxin are 7.4 and 1.1 μg, respectively. The specific neutralizing capacity obtained was of the same order for both cobra venom and cobrotoxin.

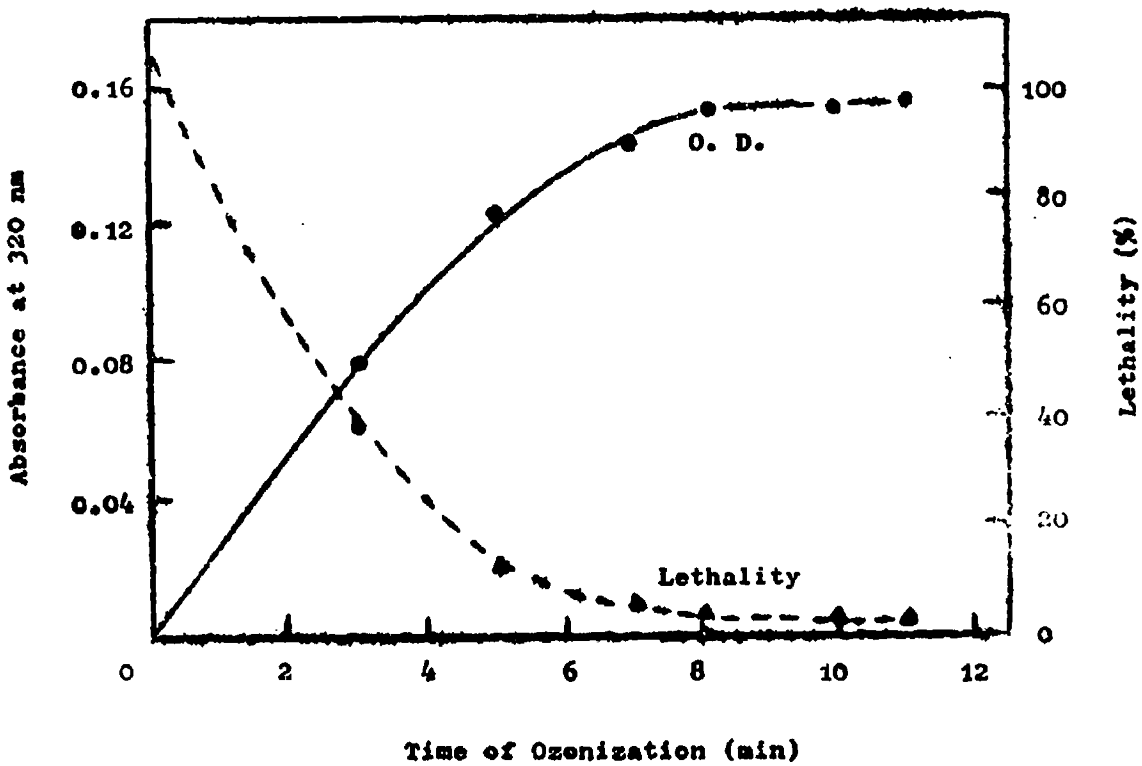


Fig. 1. Relationship between the formation of N-formylkynurenine and the lethal toxicity of cobrotoxin as a function of time of ozonization.

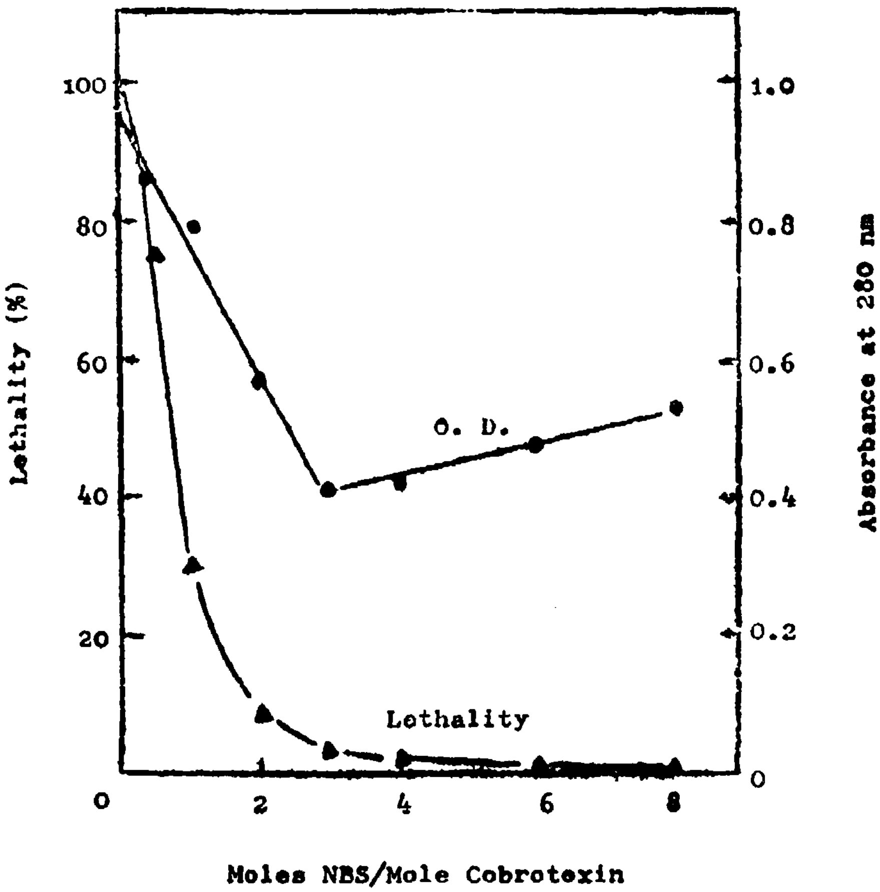
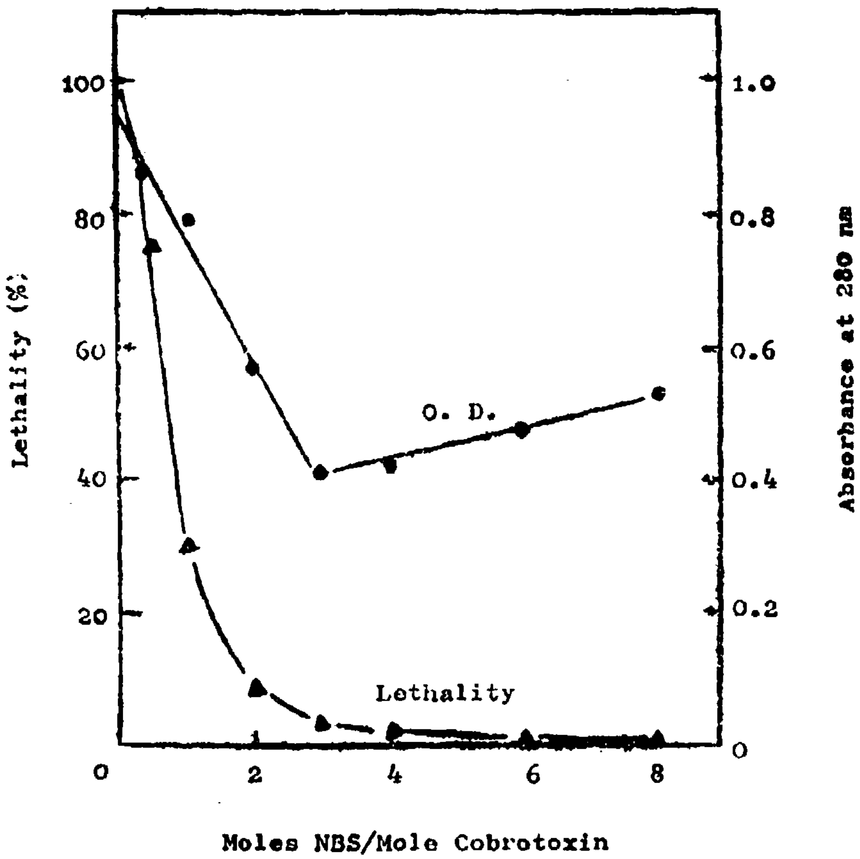


Fig. 2. Change of absorbance and decrease of lethal toxicity of cobrotoxin after oxidation with NBS.

To the solutions of cobrotoxin (3 mg) in 2 ml of 0.1 M acetate buffer (pH 4.0), varying molar equivalents of NBS in 1.0 ml of the same buffer were added dropwise. $A_{280\ nm}$ (1-cm light path) value and lethal toxicity were plotted against the amounts of NBS after the reaction had proceed for 30 min.



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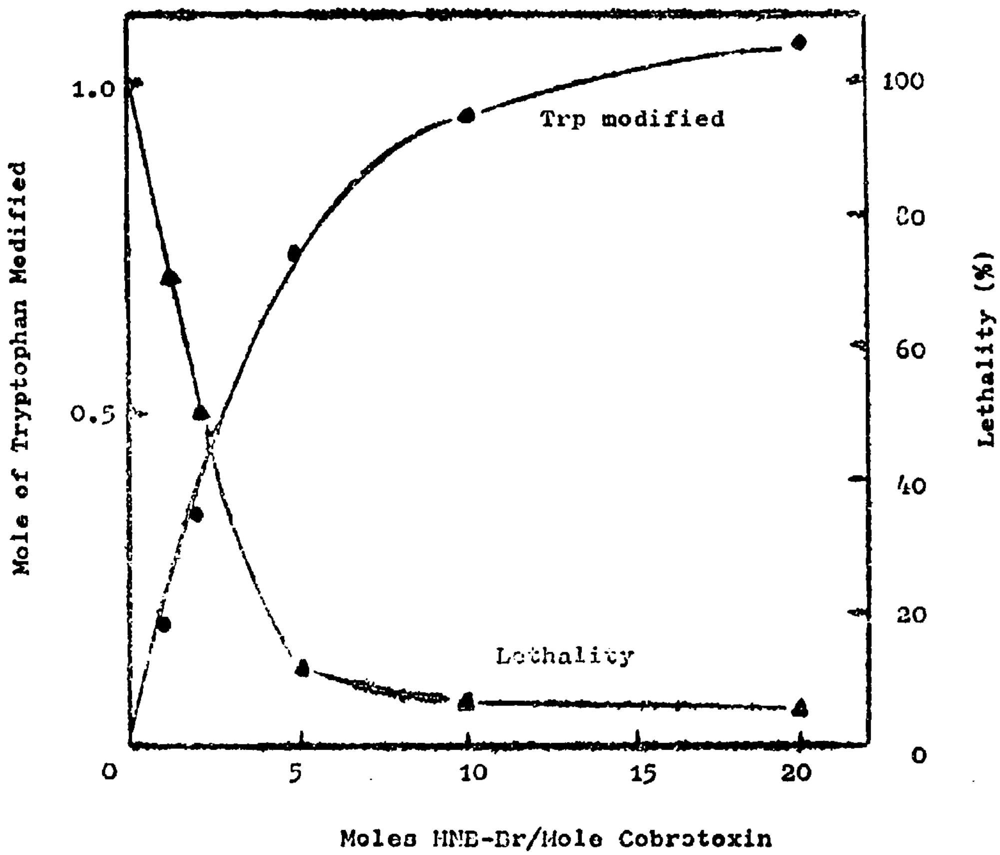

Fig. 3. Alkylation of tryptophan residue in cobrotoxin with HNB-Br and decrease of lethal toxicity.

To the solutions of cobrotoxin (1 μ mole) in 2 ml of 0.18 M acetic acid (pH 2.7), various molar excess of HNB-Br in 0.2 ml of dry acetone were added. After vigorous stirring for 1 h at room temperature (27°C), the mixture was desalted by passage through a Sephadex G-25 column (2 x 33 cm) and eluted with 0.2 M acetic acid. The protein fractions were pooled and lyophilized. The mole of tryptophan modified was determined spectrophotometrically on dry sample dissolved in 0.05 M sodium carbonate buffer (pH 10.0), using a value of 18,900 for the molar extinction coefficient at 410 nm (15).

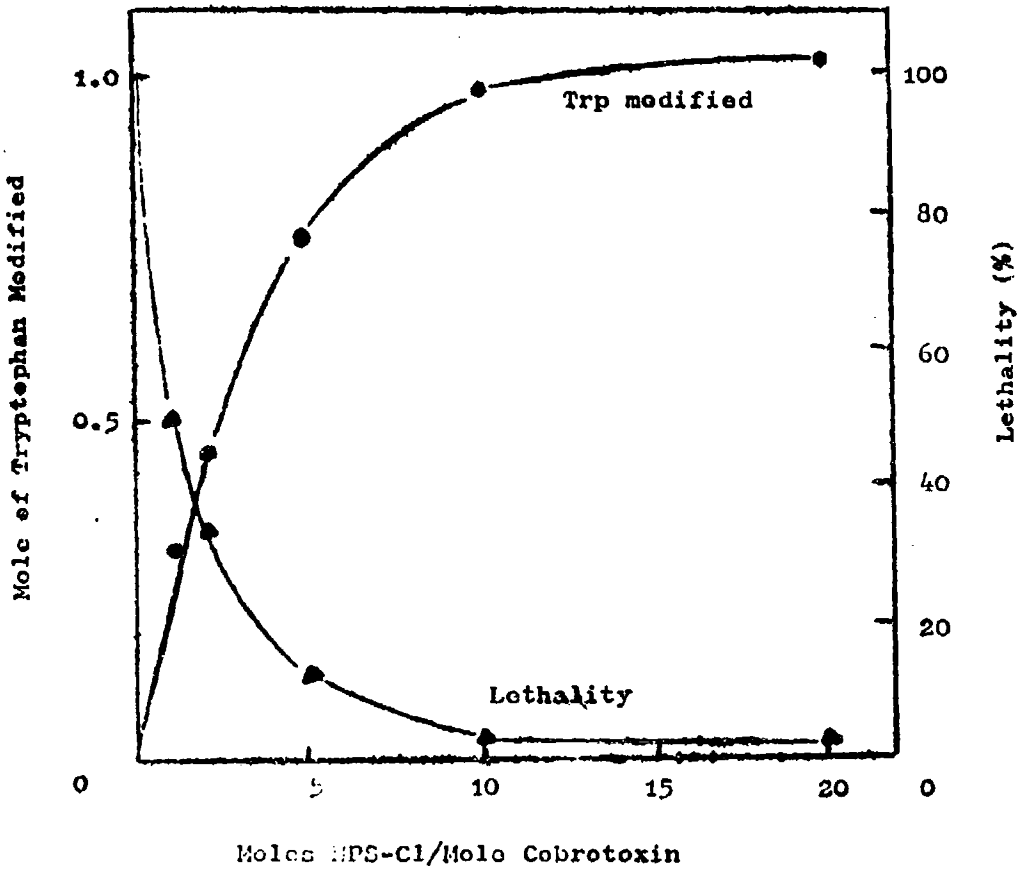

Fig. 4. Sulfenylation of tryptophan residue in cobrotoxin with NPS-Cl and decrease of lethal toxicity.

To the solutions of cobrotoxin (1 μ mole) in 2 ml of 30 % acetic acid, various molar excess of NPS-Cl in 1 ml of glacial acetic acid were added. After stirring for 1 h at room temperature (27°C), the modified toxin was separated from the reagent by passage through a Sephadex G-25 column (2 x 33 cm) and eluted with 0.2 M acetic acid. The protein fractions were pooled and lyophilized. The NPS-tryptophan was determined spectrophotometrically on the dry sample dissolved in 0.2 M acetic acid, using a value of 4,000 for the molar extinction coefficient at 365 nm (11).

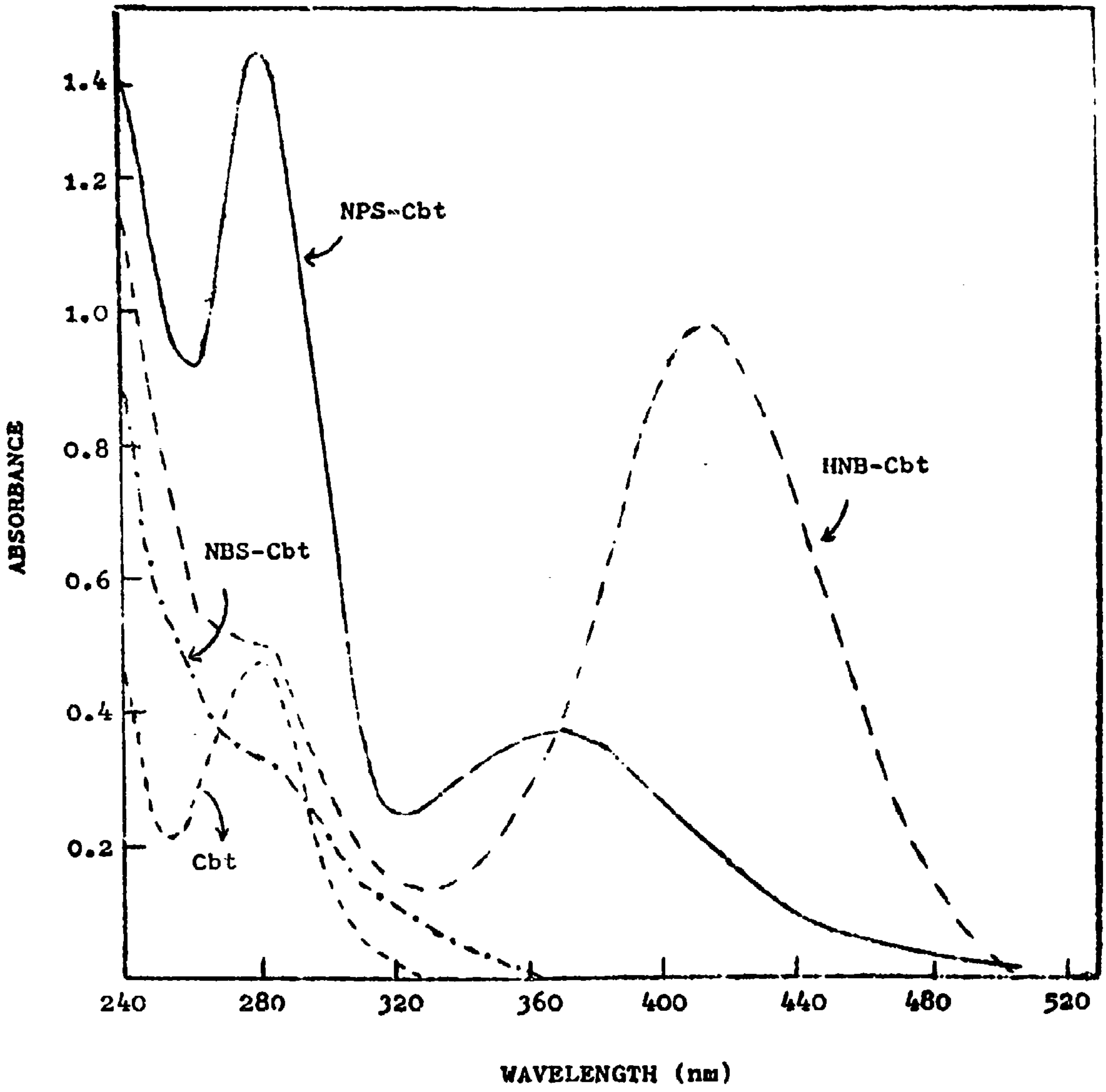

Fig. 5. Absorption spectra of cobrotoxin and its Trp-modified derivatives.

3 mg of cobrotoxin and 2 mg of modified derivatives were dissolved in 6 ml of 0.2 M acetic acid, respectively, except for HNB-cobrotoxin which was dissolved in 0.05 M sodium carbonate buffer (pH 10.0). Cbt, cobrotoxin.

APPENDIX B-6

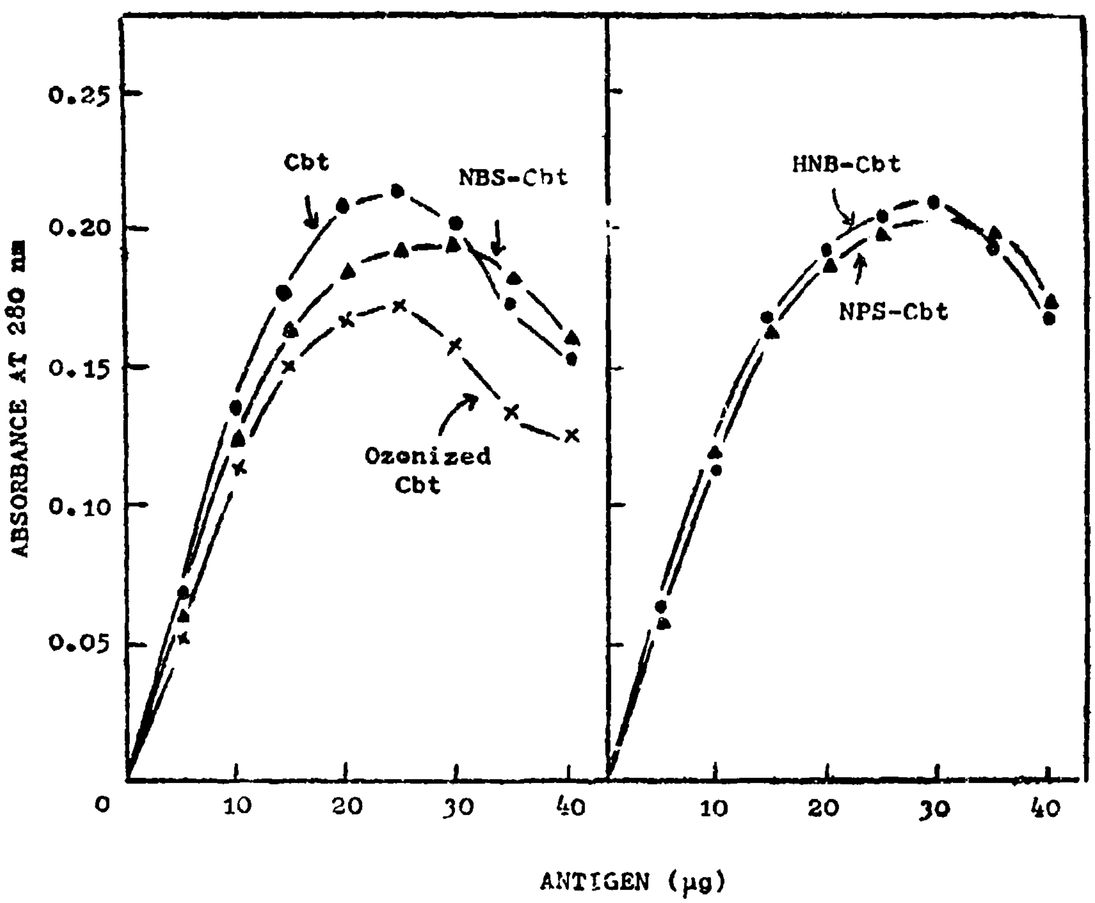

Fig. 6. Quantitative precipitin reactions of cobrotoxin and the Trp-modified derivatives with anti-cobrotoxin sera.

0.5 ml of the pooled antisera from four rabbits were used in each determination. Cbt, cobrotoxin.

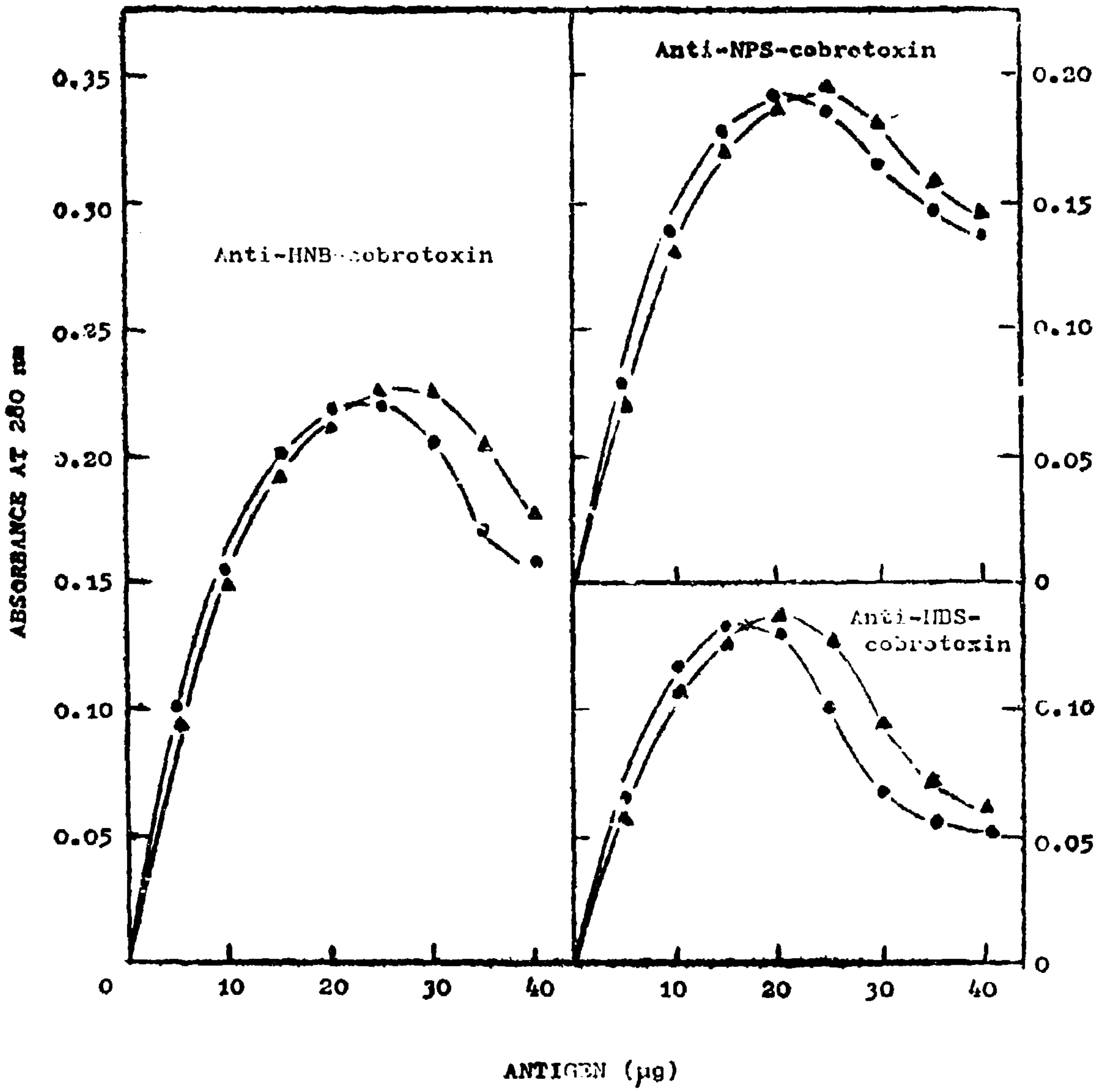

Fig. 7. Quantitative precipitin reactions of cobrotoxin and the Trp-modified derivatives with anti-Trp-modified cobrotoxin sera.

0.5 ml of the pooled antisera from four rabbits were used in each determination. ●——●, cobrotoxin; ▲——▲, homologous modified cobrotoxin.

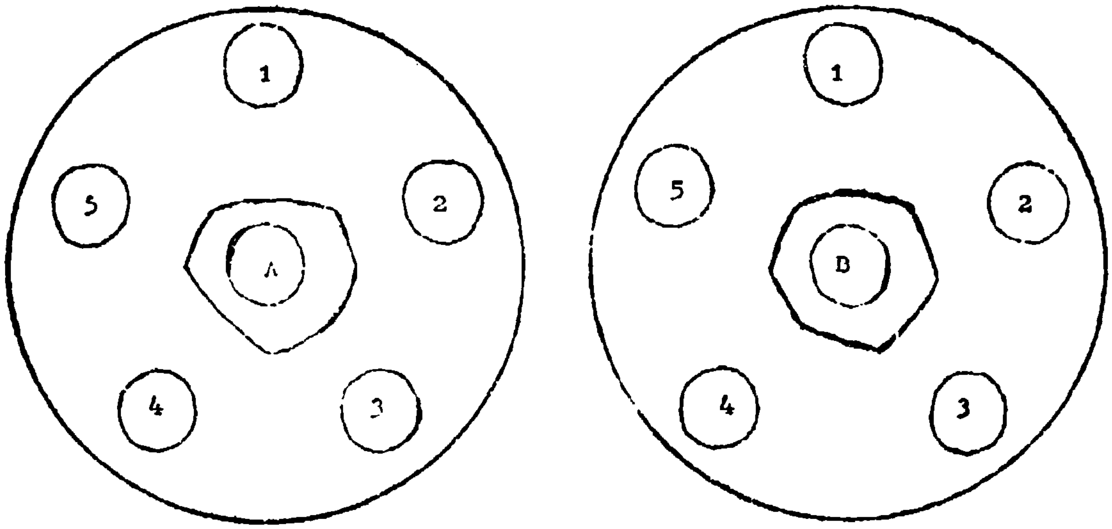

Fig. 8. Immunodiffusion in agar gel.

Central wells: (A) Anti-cobrotoxin sera; (B) Anti-HNB-cobrotoxin sera. Surrounding wells: (1) Cobrotoxin; (2) HNB-cobrotoxin; (3) NBS-cobrotoxin; (4) NPS-cobrotoxin; (5) Ozonized cobrotoxin.

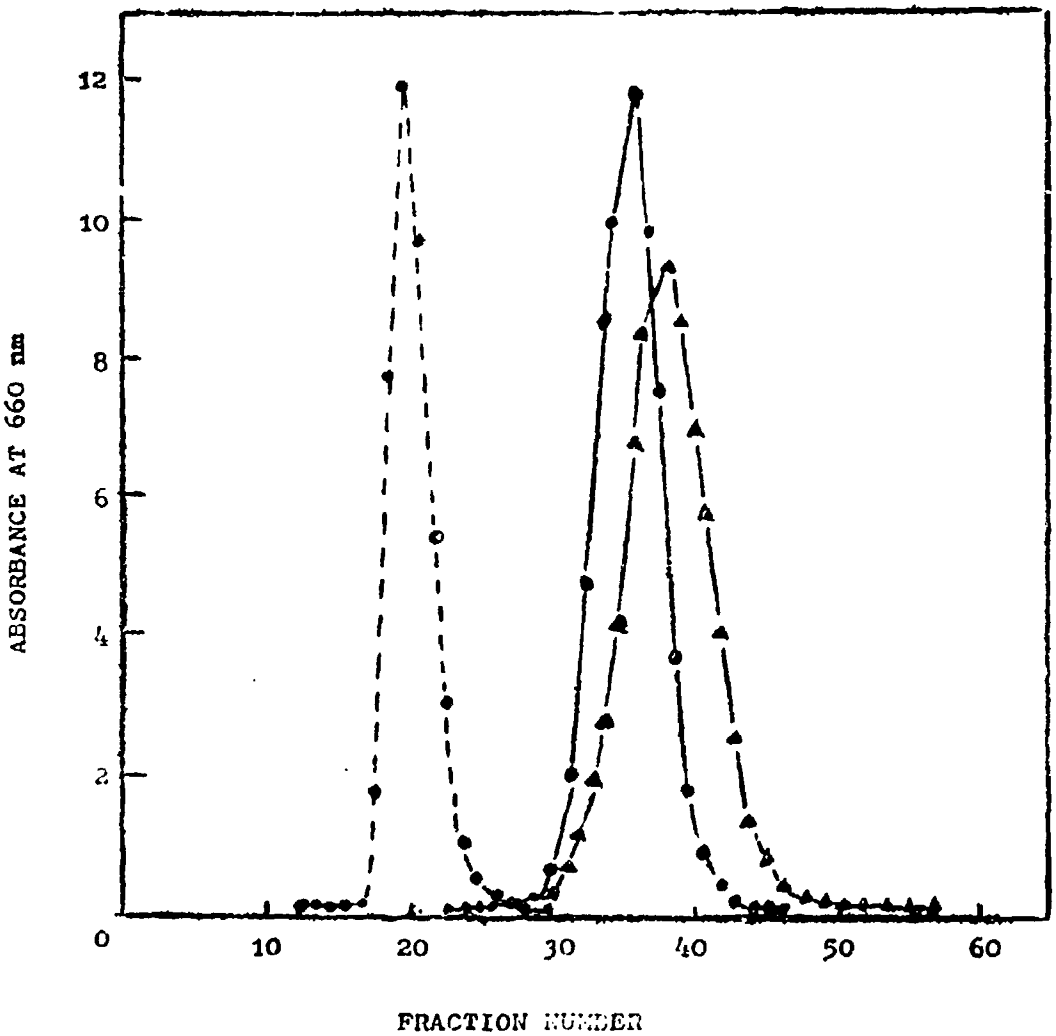

Fig. 9. Gel filtration patterns of cobrotoxin, HNB-cobrotoxin, and α-chymotrypsin on Sephadex G-50.

The column was equilibrated with M/15 phosphate buffer (pH 7.4) to the constant height (2 x 85 cm). Each sample (10 mg) dissolved in the same buffer was applied into the column and eluted with the same buffer. 5 ml of fractions were collected at a rate of 25 ml per h and the protein concentration was determined by the method of Lowry et al. (21). ●——●, cobrotoxin; ▲——▲, HNB-cobrotoxin; ●-----●, α-chymotrypsin.

List of Publications

1. The disulfide bonds of cobrotoxin and their relationship to lethality. Biochim. Biophys. Acta, 133 (1967) 346.

2. Optical rotatory dispersion of cobrotoxin. J. Biochem., 61 (1967) 272.

3. Biochemical studies on the toxic nature of snake venom. Intern. Conger. Biochem., 7th, Tokyo Col. VIII, 1 (1967).

4. Studies on fluorescent cobrotoxin. Biochim. Biophys. Acta, 147 (1967) 600.

5. Study on I^{131} labeled cobrotoxin. Toxicon, 5 (1968) 295.

6. Optical rotatory dispersion and circular dichroism of cobrotoxin. Biochim. Biophys. Acta, 168 (1968) 373.

7. Amino acid composition and end group analysis of cobrotoxin. Toxicon, 7 (1969) 43.

8. Immunochemical studies on cobrotoxin. J. Immun., 102 (1969) 1437.

9. The amino acid sequence of cobrotoxin. Biochim. Biophys. Acta, 188 (1969) 65.

10. Biochemical and immunochemical studies on cobrotoxin. The Snake, 2 (1970) 1.

11. Structure-activity relationships and immunochemical studies on cobrotoxin. Radiation Sensitivity of Toxins and Animal Poisons, IAEA-PL-334/6 (1970) 63.

12. The position of disulfide bonds in cobrotoxin. Biochim. Biophys. Acta, 214 (1970) 355.

13. **Biochemical studies on the toxic nature of snake venom: Cobrotoxin from Formosan cobra venom.** <u>Toxins of Animal and Plant Origin</u>, DeVries/ Kochva, Gordon and Breach, London, 1 (1971) 205.

14. **Studies on the status of tyrosyl residues in cobrotoxin.** <u>Biochim. Biophys. Acta</u>, 236 (1971) 164.

15. **Studies on the status of free amino and carboxyl groups in cobrotoxin.** <u>Biochim. Biophys. Acta</u>, 251 (1971) 334.

16. **Photooxidation of cobrotoxin.** <u>J. Formosan Med. Assoc.</u>, 71 (1972) 383.

17. **Iodination of cobrotoxin.** <u>Toxicon</u>, in press (1972).

18. **Immunochemical studies on the tryptophan-modified cobrotoxin.** <u>Biochim. Biophys. Acta</u>, in press (1973).

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